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**13. ABSTRACT (Maximum 200 Words)**

**G protein-coupled receptors (GPCR) are a family of receptors that are involved in all physiologic and many pathologic processes. Recently, they have been described as playing a critical role in breast cancer metastasis. As all GPCRs interact with arrestins, we have sought to better understand the role of arrestin interaction with GPCRs particularly in the process of apoptosis. Previously published data has described the initiation of apoptosis when GPCRs are stimulated in the absence of arrestins. It is therefore our goal to better understand this process and therefore better understand potential roles for GPCRs and arrestins in breast cancer development. From this work, we hypothesize that arrestin can be used as a novel chemotherapeutic target. If arrestins are inhibited, stimulation of GPCRs found on metastatic breast cancer cells may undergo apoptosis therefore providing a new treatment to eradicate breast cancer.**

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## INTRODUCTION

The purpose of this research is to understand the role of G protein-coupled receptors (GPCR) and arrestins in breast cancer metastasis. Originally, the proposal was designed to use CXCR4 as a model for our research as it has been specifically demonstrated to be involved in breast cancer metastasis (*1*). We proposed to understand arrestin's involvement in CXCR4 signaling. In addition, we hypothesized that stimulation of CXCR4 in the absence of arrestins would lead to apoptosis. This was based upon preliminary data demonstrating that *N*-formyl peptide receptor (FPR) activation in the absence of arrestin initiated apoptosis. Therefore, arrestin could be a novel chemotherapeutic target for metastatic breast cancer cells that overexpress CXCR4. However, data published shortly after the receipt of this award demonstrated that CXCR4 did not cause apoptosis in the absence of arrestin (*2*). This was a foreseen difficulty listed in the **"Potential Pitfalls"** section of my proposal. To address this difficulty, we proposed that we could change receptors and continue to study the mechanisms of GPCR/arrestin pathways that lead to apoptosis. Therefore, we have changed receptors to the FPR and will continue our studies on this receptor. This receptor will work as an excellent model for the role of GPCRs and arrestin in breast cancer as it is a member of the same receptor family, reagents are available that make the research methods easier and the research will continue to answer the same fundamental questions asked in the original proposal. **These changes have been recently submitted to the DOD in a revised Statement of Work.**

## BODY

Mutants of arrestin-2 were designed to narrow regions in the tail that regulate GPCR-mediated apoptosis (Figure 1). Some of these mutants were made based on previously known mutations of arrestin that prevent interaction with AP-2 (*3*), extracellular signal-regulated kinase (ERK) 1/2 (*4*), and clathrin (*5-7*) or were known to be constitutively active (arr2-3A) (*8*). The remainder of the mutants were designed by mutating qualitatively similar amino acids in regions of arrestin to alanine. These mutants were created by PCR mutagenesis and cloned into HindIII/ApaI sites into pEGFP-N1 vector or pmRFP1. All mutants were confirmed by DNA sequencing.

```
383     390         400         410       418
  DDDIVFED FARQRLKGMK DDKEEEEDGT GSPRLNDR - native sequence
  DDDAAAED FARQRLKGMK DDKEEEEDGT GSPRLNDR - arr2-3A-GFP
  AAAAAAAA FARQRLKGMK DDKEEEEDGT GSPRLNDR - arr2-8A-GFP
  DDDIVFED AAAAAAKGMK DDKEEEEDGT GSPRLNDR - arr2-6A-GFP
  DDDIVFED FARQRLAAAA DDKEEEEDGT GSPRLNDR - arr2-4A-GFP
  DDDIVFED FARQRLKGMK AAAAAAAAGT GSPRLNDR - arr2-8A'-GFP
  DDDIVFED FARQRLKGMK DDKEEEEDGT GAPRLNDR - arr2-5A-GFP
  DDDIVFED FARQRLKGMK DDKEEEEDAA AAAAAAAA - arr2-10A-GFP
  DDDIVFED FARQRLKGMK DDKEEEEDGT GDPRLNDR - arr2-S412D-GFP
  DDDIVFED AARQRLKGMK DDKEEEEDGT GSPRLNDR - arr2-F391A-GFP

  -There is a clathrin sequence just before this region (LIELD)
        that has also been deleted - arr2-ΔLIELD-GFP.
```

**Figure 1. Map of arrestin-2 and mutants generated by PCR mutagenesis.** Arrestin mutants were designed based on previously known mutations or by changing qualitatively similar amino acids to alanine. Mutants were generated with PCR mutagenesis and subcloned into pEGFP-N1 or mRFP1.

Our lab has demonstrated the importance of arrestin in preventing GPCR-mediated apoptosis. We have narrowed the regions that may be responsible by analyzing transiently transfected arrestin knockout mouse embryonic fibroblasts stably transduced with the FPR (KOFPR) for uptake of propidium iodide after stimulation (5hr) with 633-6pep (agonist) or serum-free medium (SFM) alone. Three hundred arrestin mutant-expressing KOFPRs (green) were randomly viewed via confocal fluorescence

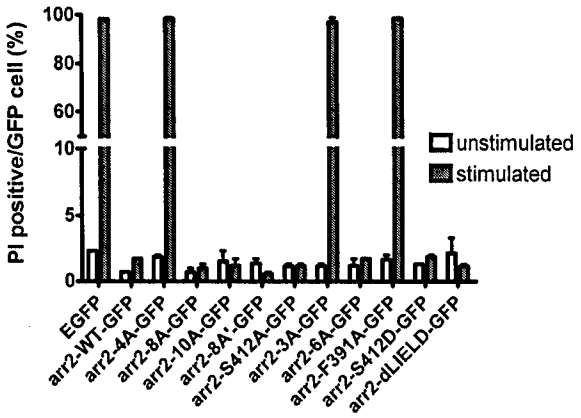


**Figure 2. Regions of arrestin that are responsible for FPR-mediated apoptosis.** Transiently transfected KOFPRs were plated on glass coverslips and stimulated with 10nM 633-6pep for five hours. Cells were stained with 100pg/μL PI, washed, fixed, mounted and assayed for percentage PI positive cells/GFP expressing cells. Data expressed as mean ±SEM and are representative of two independent experiments.

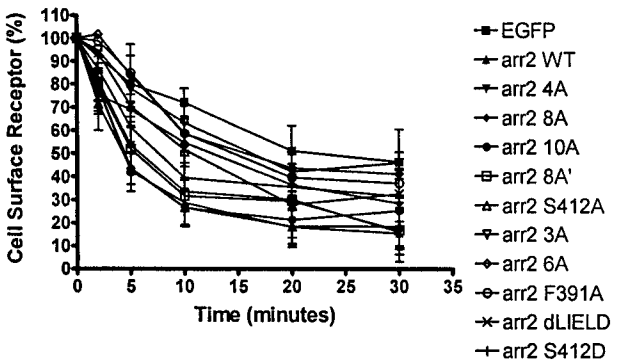


**Figure 3. Internalization of the FPR in presence of arrestin-2 mutants.** Transiently transfected KFs were stimulated with 1μM fMLF, aliquoted at time points shown, washed 3 times with SFM and labeled with 633-6pep for analysis by flow cytometry. Data are expressed as mean±SEM and are representative of three independent experiments.

microscopy. Those cells were noted to be propidium iodide positive or negative. Results are shown in Figure 2 and are represented as the percentage of PI positive cells per GFP positive cell. Empty GFP vector or arrestin-2-WT-GFP were used as controls. In addition, KOFPRs and wild-type littermates stably expressing the FPR (WTFPR) were assayed as well and are consistent with previously published results (2).

To determine whether FPR internalization was required to generate apoptosis we assayed the internalization of the FPR in the presence of GFP-fused arrestin-2 mutants generated above. KOFPRs transiently transfected with GFP-fused arrestin-2 mutants were assayed for FPR internalization. The FPR internalizes in the presence of arrestin-2 mutants as well or better than (EGFP) empty vector (Figure 3). At this time, we conclude that any arrestin-2 mutant that will inhibit FPR-mediated apoptosis is doing so for some other reason than preventing the FPR from leaving the cell surface. Untransfected KOFPRs and WTFPRs were also assayed and consistent with previous published results (9).

Previous results have demonstrated that in stimulated KOFPRs, the FPR accumulates in a perinuclear location (9). This result was further described as being a Rab11-positive endosome indicative of a recycling compartment. The report also noted that the result corresponded with an inability of the receptor to recycle. We sought to understand the relationship between arrestin-2 mutants that do not rescue apoptosis and their trafficking with respect to Rab11. Experiments were conducted in which KOFPRs were transiently transfected with Rab11-WT-GFP and various RFP-fused arrestin mutants. Transfected cells were then plated on glass coverslips, stimulated for 1 hour with 633-6pep, fixed and mounted. Cells were imaged by confocal fluorescence microscopy. As can be seen in Figure 4, in KOFPRs transfected with mRFP vector (empty), the FPR accumulates in the Rab11-positive endosome in a perinuclear location. In cells transfected with wild-type arrestin, the receptor/arrestin complex is found colocalized with Rab11, but significant amounts are also found in other, more peripheral areas of the cell. This is indicative of the receptor's ability to traffic normally.

Also, the arrestin-2-4A mutant (which does not rescue apoptosis) behaves similarly to arrestin-deficient cells with respect to FPR trafficking while the arrestin-2-S412D mutant (which does rescue apoptosis) behaves like cells expressing wild-type arrestin. The remainder of the arrestin mutants behave in a manner consistent with their ability to rescue FPR-mediated apoptosis. Therefore, normal trafficking of the FPR appears to predict arrestin's ability to rescue apoptosis.

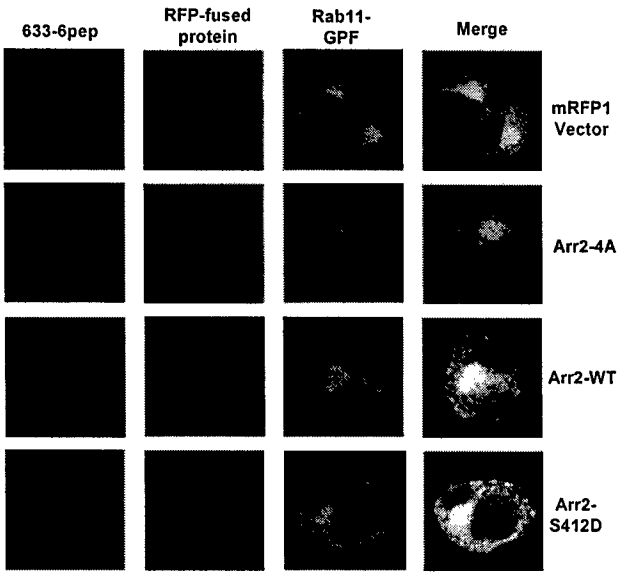


**Figure 4. Arrestin mutants incapable of rescuing apoptosis accumulate in a Rab11-positive, perinuclear location.** Transiently transfected KOFPRs were plated and glass coverslips, stimulated with 633-6pep for 1 hour, fixed, mounted and viewed by confocal fluorescence microscopy. Representative images are shown and are indicative of one experiment.

## KEY RESEARCH ACCOMPLISHMENTS

- Arrestin-2 mutants have been constructed
- We have found mutants that are responsible for FPR-mediated apoptosis
- We have seen that arrestin effect on apoptosis is independent of their effect on FPR internalization
- We have demonstrated that trafficking patterns of arrestin mutants that do not inhibit apoptosis are similar to when no arrestin is present.
- We have demonstrated that trafficking patterns of arrestin mutants that inhibit apoptosis are similar to when wild-type arrestin is present.
- We have realized that arrestin mutants that do not inhibit apoptosis all fail to interact with AP-2 (a regulator of receptor trafficking) and are investigating its role on GPCR/arrestin interactions and apoptosis.

## REPORTABLE OUTCOMES

- Presented poster on current research at the American Society for Cell Biology Conference in Washington, DC—December 2004.
- Awarded Edmund J. and Thelma W. Evans Charitable Trust Scholarship
- Awarded Biomedical Sciences Graduate Program Travel Award
- Awarded Office of Graduate Studies Travel Award
- Passed Comprehensive Exam with Distinction

## CONCLUSIONS

These results demonstrate that there are specific regions of arrestin responsible for controlling FPR-mediated apoptosis. In addition, trafficking of the receptor/arrestin complex is important to the initiation of apoptosis and may be regulated by AP-2. Future work will address the signaling differences in arrestin mutants that differentially regulate apoptosis and whether these processes take place in breast cancer cells.

These findings are important as GPCRs have been shown to be overexpressed in cancer cells (most notably breast cancer) and more are likely to be found. As all GPCRs interact with arrestin, understanding GPCR/arrestin interaction is crucial to understanding the role of GPCRs in metastatic breast cancer cells. GPCRs are not only involved in metastasis, but migration of metastatic cells is aided by these receptors. Understanding the role of arrestins with GPCRs could lead to novel chemotherapeutic therapy for breast cancer metastasis that may reduce metastasis by inhibiting migration or inducing apoptosis in breast cancer cells overexpressing GPCRs.

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